

## Comparison of Direct Examination and *In Vitro* Culture for the Detection of *Blastocystis* sp. in Orang Asli Stool Samples

(Perbandingan Pemeriksaan Langsung dan Kultur *In Vitro* untuk Pengesanan *Blastocystis* sp. dalam Spesimen Najis Orang Asli)

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### ABSTRACT

*Microscopy-based technique has been widely used in the detection of Blastocystis sp. This study was conducted to compare the techniques used for screening of Blastocystis sp., namely in vitro cultivation of stool specimens in Jones' medium (IVC) followed by Wheatley Trichrome staining and direct examination of stool samples preserved with polyvinyl alcohol (PVA) followed by Wheatley Trichrome staining with single-round polymerase chain reaction (PCR) as the reference technique. The study was performed on 466 stool samples obtained from the aboriginal community in Pahang, Malaysia. IVC showed higher detection rate of Blastocystis sp. (35.6%) than PVA (20.0%). Single-round PCR detected Blastocystis sp. in 41.0% of the stool specimens. The sensitivity and specificity of PVA and IVC in comparison to the reference technique were 75.3% (95% CI: 65.2-83.6) and 68.5% (CI: 63.7-73.3) and 88.6% (CI: 82.7-93.0) and 86.3% (CI: 81.9-90.0), respectively. The agreement between the reference technique and PVA showed statistically significant fair agreement by Cohen Kappa statistics of (K=0.318, p<0.001), meanwhile statistically significant substantial agreement was observed between PCR and IVC by Cohen Kappa (K=0.727, p<0.001). Therefore, in vitro cultivation in Jones' medium followed by Wheatley Trichrome staining of stool specimens should be used as a screening technique in the detection of Blastocystis sp. infections.*

**Keywords:** *Blastocystis* detection; in vitro cultivation; microscopy; polymerase chain reaction; polyvinyl alcohol

### ABSTRAK

*Teknik berasaskan mikroskopi telah digunakan secara meluas dalam pengesanan Blastocystis sp. Kajian ini dijalankan untuk membandingkan dua teknik saringan; kultur secara in vitro spesimen najis dalam media Jones' (IVC) diikuti dengan pewarnaan Trikom Wheatley dan pemeriksaan langsung sampel najis yang diawet dengan alkohol polivinil (PVA) diikuti dengan pewarnaan Trikom Wheatley dengan satu pusingan tindak balas berantai polimerase (PCR) sebagai teknik rujukan. Kajian ini dijalankan terhadap 466 sampel najis yang diperolehi daripada komuniti Orang Asli di Pahang, Malaysia. Teknik IVC menunjukkan kadar pengesanan yang lebih tinggi (35.6%) berbanding PVA (20.0%). Kesensitifan dan kekhususan PVA dan IVC berbanding teknik rujukan masing-masing adalah 75.3% (95% CI: 65.2-83.6) dan 68.5% (CI: 63.7-73.3) dan 88.6% (CI: 82.7-93.0) dan 86.3% (CI: 81.9-90.0). Darjah persetujuan antara teknik rujukan dan PVA menunjukkan persetujuan sederhana menggunakan analisis Cohen Kappa (K=0.318, p<0.001), manakala persetujuan yang baik ditunjukkan antara PCR dan IVC menggunakan analisis yang sama (K=0.727, p<0.001). Oleh yang demikian, teknik kultur secara in vitro dalam media Jones' diikuti pewarnaan Trikom Wheatley untuk spesimen najis harus digunakan sebagai teknik saringan dalam pengesanan jangkitan Blastocystis sp..*

**Kata kunci:** Alkohol polivinil; kultur secara in vitro; mikroskopi; pengesanan *Blastocystis*; tindak balas berantai polimerase

### INTRODUCTION

*Blastocystis* sp. is a single-celled protist which has worldwide distribution with higher prevalence rates in developing countries (Graczyk et al. 2005). Detection of *Blastocystis* sp. in humans is usually based on microscopic examination of stool samples (Eida & Eida 2008). Direct microscopy is usually performed with either in wet mount preparations or stained stool specimens. Stains used in *Blastocystis* sp. detection include Lugol's iodine for wet mount preparations and permanent stains including Wheatley Trichrome, Giemsa, acid-fast and Field's. Among

the stains used, Wheatley Trichrome has been the most practiced in many clinical parasitology laboratories as it is found to be more sensitive in the detection of *Blastocystis* sp. and many other intestinal protozoa than wet mount preparations stained with Lugol's iodine. Detection rate of *Blastocystis* sp. improves greatly by microscopy with Wheatley Trichrome stain or when high quality optics are applied (Hazen 1993; Lee 1991; Markell & Udkow 1990). In Malaysia, almost all microscopic techniques in *Blastocystis* sp. detection used either direct smear of stool, formalin ethyl acetate concentration, staining or

wet smear of *in vitro* cultivation product (Abdulsalam et al. 2012; Ghani & Yusof 2011; Hanapian et al. 2014; Ishak et al. 2008).

*Blastocystis* sp. portrays pleomorphic forms including vacuolar, multivacuolar, amoeboid, cyst and granular which vary in sizes. However, it may be difficult to distinguish *Blastocystis* sp. in different forms from leucocytes or trophozoites or cyst of other protozoa. The pleomorphic forms of *Blastocystis* sp. poses identification and diagnostic problems (Eida & Eida 2008). Therefore, several diagnostic approaches should be used in order to get higher detection of *Blastocystis* sp. in stool specimens (Tan et al. 2008). A reliable diagnosis is important in choosing appropriate microscopic technique (van Lieshout & Roestenberg 2015). The aim of this study was to compare fixed-polyvinyl alcohol stool specimens followed by Wheatley Trichrome staining and *in vitro* cultivation of stool specimens in Jones' medium followed by Wheatley Trichrome staining in the detection of *Blastocystis* sp. in reference to polymerase chain reaction. To the best of our knowledge, this will be the first study to performed comparison on both techniques.

## MATERIALS AND METHODS

### SAMPLES

Fresh stool samples obtained from the Orang Asli community in Temerloh, Pahang Malaysia were divided into three aliquots. Approximately 1/3 of the collected stool was subjected to *in vitro* cultivation (IVC) in Jones' medium, another 1/3 was placed in polyvinyl alcohol (PVA) and the remaining 1/3 stool was stored at 0°C for molecular characterization of *Blastocystis* sp. by DNA extraction and polymerase chain reaction.

### MICROSCOPY-BASED TECHNIQUES

**PVA** Stool specimens fixed in polyvinyl alcohol were stained using Wheatley Trichrome stain (PVA) with the followings; 70% alcohol with several drops of Tincture iodine (10-15 min), 70% alcohol (1 min), 70% alcohol (1 min), Trichrome stain (8-10 min), Acid alcohol (2-4 s), 95% alcohol (1 min), absolute alcohol (1 min) and Wintergreen oil (5 min) (Salleh et al. 2012). Positivity was cross-checked by experienced laboratory technician.

**IVC** A pea-sized of the second portion of the fresh stool samples were inoculated into 3 mL Jones' medium supplemented with 10% horse serum and incubated at 37°C for 5 days (Farah et al. 2014). Approximately 100 µL of the cultured-products were smeared on a cover slip and air-dried for 24 h. The smeared cover slip was then subjected to Wheatley Trichrome staining as mentioned before.

### MOLECULAR-BASED TECHNIQUE (REFERENCE TECHNIQUE)

DNA of *Blastocystis* sp. from the stool of Orang Asli were extracted using QIAmp®DNA stool minikit (Qiagen,

Germany) according to the manufacturer's protocol. Screening of all samples were carried out using primer complementary to the SSU rDNA sequence from the samples using BhrDr and RD5 primers (Scicluna et al. 2006). PCR amplification was performed using Eppendorf Pro-S thermal cycler (Hamburg, Germany) by 30 cycles of initial denaturation at 95°C for five min, followed by denaturation at 95°C for one min, annealing at 63.3°C for one min and 30 s, extension at 72°C for one min and an additional cycle of 10 min chain elongation at 72°C. The PCR products were separated in 1.5% agarose gel. Positive PCR products were sent for sequencing to obtain subtypes information based on comparisons with the data available in Genbank using BLASTN program (<http://www.ncbi.nlm.nih.gov/BLAST>).

## RESULT AND DISCUSSION

Positivity of *Blastocystis* sp. using PVA and IVC was dependent upon the presence of any of the four morphologies (vacuolar, granular, cyst and amoeboid) (Figures 1 & 2). Meanwhile, sample was considered positive when there was a band at ~600 bp on the agarose gel and confirmed by sequencing (Figure 3). The prevalence rates of *Blastocystis* sp. in all examined stool specimens using three different diagnostic techniques were 35.6% (IVC), 20.0% (PVA) and 41.0% (PCR) (Table 1). Hundred seven-teen (61.3%) and forty one (21.5%) stool specimens from Orang Asli were negative by PVA and IVC, respectively, proved to be positive by PCR assay. However, single-round PCR was unable to amplify forty two (15.3%) stool specimens among Orang Asli which was initially diagnosed by microscopic diagnostic methods (PVA and IVC). Sequencing of 191 positive PCR-product of Orang Asli stool specimens showed *Blastocystis* ST1, ST2, ST3 and ST4.

Sensitivity, specificity, PPV and NPV of microscopic techniques used in the detection of *Blastocystis* sp. in comparison to single-round PCR were presented in Table 2. The sensitivity and specificity of PVA and IVC compared to the reference technique were 75.3% (95% CI: 65.2-83.6) and 68.5% (CI: 63.7-73.3) and 88.6% (CI: 82.7-93.0) and 86.3% (CI: 81.9-90.0), respectively. The agreement between the reference technique and PVA showed fair statistically significant agreement by Cohen Kappa statistics of ( $K=0.318$ ,  $p<0.001$ ), meanwhile substantial statistically significant agreement was observed between PCR and IVC by Cohen Kappa ( $K=0.727$ ,  $p<0.001$ ).

Microscopy technique is the gold standard for the diagnosis of almost all intestinal protozoan infections. Microscopic techniques that usually used in parasitology laboratory for the identification of *Blastocystis* sp. include wet preparation and permanently stained of stool specimens. Among various known permanent stain used to aid in microscopic observation was Wheatley Trichrome. This stain is routinely used in many clinical microbiology laboratories and has shown to be more

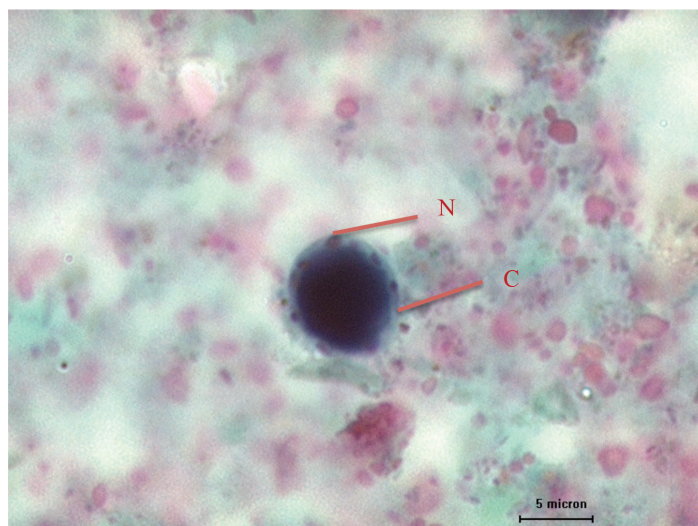


FIGURE 1. Vacuolar form detected by PVA and stained with Trichrome in isolates from a diarrhoeic subject with large central vacuole (V), many nucleus (N) in the cytoplasm (C)

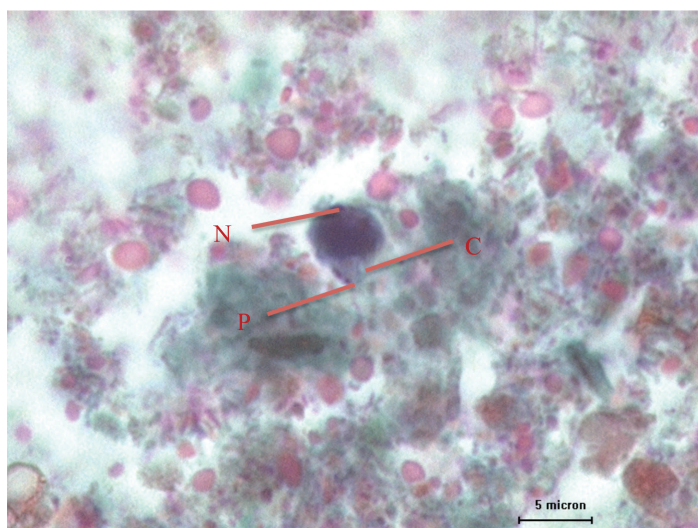


FIGURE 2. Amoeboid form of *Blastocystis* sp. detected using IVC and stained by Trichrome in isolates from the same diarrhoeic subject with many nucleuses at the rim of cytoplasm.

P: pseudopod-like projection, N: nucleus, C: cytoplasm. This particular sample was positive for infection by *Blastocystis* sp. ST3 detected by PCR



FIGURE 3. Results of the single-round PCR for DNA extracted from human stool samples

TABLE 1. Comparison of different diagnostic techniques in the detection of *Blastocystis* sp. infections

Diagnostic techniques	Positive (%)	Negative (%)
Wheatley Trichrome staining of fixed PVA stool samples	93 (20.0)	373 (80.0)
Wheatley Trichrome staining of IVC in Jones' medium	166 (35.6)	300 (64.4)
Single-round PCR followed by sequencing <sup>a</sup>	191 (41.0)	275 (59.0)

<sup>a</sup>Specific for *Blastocystis* sp. subtypes: ST1, ST2, ST3, ST4

TABLE 2. Sensitivity and specificity of PVA followed by Wheatley Trichrome staining and IVC followed by Wheatley Trichrome staining against the reference technique (single-round PCR)

	PVA followed by staining		IVC followed by staining	
	Positive	Negative	Positive	Negative
Positive by PCR (n= 191)	70	117	147	41
Negative by PCR (n= 275)	23	256	19	259
Total	93	373	166	300
Sensitivity (%)	75.3		88.6	
95% CI	65.2 – 83.6		82.7 – 93.0	
Specificity (%)	68.6		86.3	
95% CI	63.7 – 73.3		81.9 – 90.0	
PPV (%)	37.4		78.2	
NPV (%)	91.8		93.2	
Accuracy (%)	70.0		87.1	
Kappa	K=0.318		K=0.727	
(p-value)	p<0.001		p<0.001	

sensitive in the detection of intestinal parasites than wet mount preparations stained with iodine (Gardner et al. 1980; Kellogg & Elder 1999).

However, despite of its wide usage in the diagnosis of *Blastocystis* sp., the microscopy technique has few disadvantages; besides its low sensitivity, an unexperienced individual is unable to identify the great morphological diversity of this protozoa (vacuolar, granular, amoeboid and cyst) and to differentiate it with other protozoa, human cells and artefacts. The low numbers of this parasite in the stool specimens which reflected in low sensitivity of microscopy examination of direct stool smear can be improved by *in vitro* cultivation of stool specimens in culture medium. *In vitro* cultivation increases the detection rate and sensitivity of *Blastocystis* sp. in comparison to Wheatley Trichrome stained of stool smears (Roberts et al. 2011; Stensvold et al. 2007; Termmathurapoj et al. 2004). Of all the microscopy-related methods, xenic *in vitro* cultured-product (IVC) is considered the gold standard method for *Blastocystis* sp. detection (Poirier et al. 2011). IVC has been reported as more efficient in the detection of *Blastocystis* sp. than Wheatley Trichrome staining of direct stool smear although no significant difference between *in vitro* cultivation in Jones' medium and Wheatley Trichrome staining were observed (Termmathurapoj et al. 2004). Culture medium of choice in this present study was Jones' medium, a medium

which has been widely used in many previous studies to cultivate *Blastocystis* sp. (Parkar et al. 2007; Stensvold et al. 2007).

In this present study, IVC in Jones' medium followed by Wheatley Trichrome staining was not only descriptively able to detect more number of *Blastocystis* sp. infection, but this method also showed significantly higher detection rate of *Blastocystis* sp. infection in comparison to fixed-PVA stool specimens (PVA) followed by Wheatley Trichrome staining. The sensitivity of IVC and PVA followed by Wheatley Trichrome stain with PCR as reference method was 88.6% and 75.3%, respectively. The specificity of IVC was also higher (86.3%) than PVA (68.6%). Meanwhile, the negative predictive values of IVC suggested the detection of true blastocystosis-free was 93.2% in comparison to 91.8% with PVA. Meanwhile, the positive predictive value to determine the probability of having blastocytosis was 78.2% for IVC and 37.4% for PVA. Collectively, our present findings showed that IVC followed by Wheatley Trichrome stain was more superior in *Blastocystis* sp. detection in comparison to PVA followed by Wheatley Trichrome stain. However, no similar study has been performed before, therefore no confirmation can be warranted. Superiority of *in vitro* cultivation may have resulted from multiplication of *Blastocystis* sp. in the culture medium (Poirier et al. 2011). In addition to that, in this present study, Wheatley Trichrome stain was performed during the last day of



incubation of the cultured samples which may have aided in the detection of small size cysts or small vacuolar forms. Cysts of *Blastocystis* sp. are common in stool samples of infected patients, however due to their small size; this form is often missed during microscopy, hence leads to significant underestimation of *Blastocystis* sp. detection rate (Rene et al. 2009; Suresh & Smith 2004).

In the attempt to determine the epidemiology and dynamic transmission of *Blastocystis* sp. infection in a community, a highly sensitive detection method is required (Poirier et al. 2011). Polymerase chain reaction (PCR) has been identified as the most effective method in the detection of *Blastocystis* sp. infection (Roberts et al. 2011). In this present study PCR assay and PCR-product sequencing was used as a reference technique to detect and differentiate various *Blastocystis* sp. subtypes. The result showed 41.0% (191/466) of the specimens were positive for *Blastocystis* sp.; 70 specimens detected by PVA and 147 specimens detected by IVC were amplified by single round PCR and later differentiated into subtypes. In line with that, this present study concurred with the previous study findings since we detected the highest number of *Blastocystis* sp. infection using PCR technique (Parker et al. 2007; Roberts et al. 2011). PCR was used as reference technique in this present study due to its high sensitivity; besides that the primary advantages of using this technique is the ability of this technique to discriminate different *Blastocystis* sp. subtypes. These findings are useful in the understanding of epidemiology and dynamic transmission of *Blastocystis* sp. in a community. However, despite of its sensitivity, several limitations including the needs of high-tech laboratory, expensive reagents and equipment, as well as labour-intensive of the DNA extraction tend to make other cheaper, conventional method such as microscopy and permanent stain still being practised (Meurs et al. 2017; Roberts et al. 2011).

In conclusion, this present study reaffirmed the need to perform IVC followed by Trichrome staining as the screening tools for the detection of *Blastocystis* sp. especially in community laboratories that are usually not equipped with the more sensitive but costly method, which is molecular technique (PCR). High specificity of IVC followed by Trichrome stain indicated that this technique is able to discriminate individuals infected with *Blastocystis* sp. and free-*Blastocystis* sp.. Implementation of a method with relatively high sensitivity and specificity at a low cost and easy to be performed makes this technique a reliable technique for screening of *Blastocystis* sp.. PCR showed high sensitivity and specificity in the identification of *Blastocystis* sp.; therefore, it potentially will become the 'gold standard' by which other diagnostic techniques are measured.

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